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# **Prion Protein Expression Alters APP Cleavage without Interaction with BACE-1**

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## **ABSTRACT**

The prion protein (PrP) and the beta-site amyloid precursor protein (APP) cleaving enzyme 1 (BACE-1) are both copper binding proteins, but are associated with two separate neurodegenerative diseases. The role of BACE-1 in the formation of beta-amyloid has made it a major target in attempts to reduce the formation of beta-amyloid in Alzheimer's diseases. However, the suggestion that PrP, normally associated with prion diseases binds to BACE-1 and reduces its activity has led to the suggestion that the study of PrP and this interaction could be of considerable importance to Alzheimer's disease. We therefore undertook to investigate the possible interaction of these two proteins physically and at the level of transcription, translation and APP cleavage. Our findings suggest that mature PrP and BACE-1 do not physically interact, but that altered PrP expression results in altered BACE-1 protein expression and promoter activity. Additionally, overexpression of PrP results in increased cleavage of APP in contrast to previous suggestion suggesting a reduction. Our findings suggest that any relation between PrP and BACE-1 is indirect. Altered expression of PrP causes changes in the expression of many other proteins which may be as a result of altered copper metabolism.

## INTRODUCTION

Research into aspects of Alzheimer's disease has recently focused on the potential role of the prion protein (PrP)(Balducci et al., 2010; Chen et al., 2010; Forloni and Balducci, 2011; Gimbel et al., 2010; Lauren et al., 2009; Parkin et al., 2007). PrP is a cell surface metalloprotein expressed most highly in neurons(Brown et al., 1997; Sales et al., 1998). PrP is largely known for the association of an altered conformation with another family of neurodegenerative diseases, the prion diseases(Prusiner, 1998). The exact function of PrP has been frequently disputed although the majority of data suggests a role in cellular viability and possibly in antioxidant defence (Brown, 2001). While this aspect of its cell biology is unresolved, the range of other proteins that interact with PrP continues to increase. Some of these proteins have little credibility such as intracellular proteins like HAX-1(Jing et al., 2011), while others may be associated with internalisation of the protein such as LRP-1(Parkyn et al., 2008). Among these proteins are also  $\beta$ -amyloid (Lauren et al., 2009), the amyloid precursor protein (APP) and  $\beta$ -site APP cleaving enzyme (BACE-1)(Parkin et al., 2007). These proteins are all associated with Alzheimer's disease. Therefore, the possibility that interactions between these protein and PrP may alter susceptibility to Alzheimer's disease has fuelled increased interest in PrP expression as a possible risk factor.

Alzheimer's disease is the most common dementing neurodegenerative disease(Selkoe, 2011). Its exact cause remains unknown, but the close association between the disease and deposition of  $\beta$ -amyloid in the brain suggests that this protein fragment could be the causal agent(Tanzi and Bertram, 2005). The  $\beta$ -amyloid protein fragment is generated by sequential cleavage of APP by BACE-1 and  $\gamma$ -secretase.

Inherited mutations in both APP and proteins associated with its cleavage are associated with inherited forms of Alzheimer's disease(Selkoe and Podlisny, 2002).

BACE-1 is a  $\beta$ -secretase and is one of the two enzymes required for the formation of  $\beta$ -amyloid(Cole and Vassar, 2008; Howlett et al., 2000). This protein is a transmembrane aspartyl protease which is active as a homodimer and binds copper as a possible cofactor(Angeletti et al., 2005). An enzyme complex ( $\gamma$ -secretase) cleaves APP at the C-terminal end of  $\beta$ -amyloid. The amyloidogenic pathway requires BACE-1 or another  $\beta$ -secretase but this is a low frequency event in the processing of APP which is predominately cleaved by an  $\alpha$ -secretase such as ADAM10. However, the frequency at which APP enters the amyloidogenic pathway and is cleaved by BACE-1 is considered potentially one of the key determinants of the likelihood of an individual developing Alzheimer's disease. Therefore, factors that regulate the frequency at which BACE-1 cleaves APP are of considerable importance(Citron, 2004).

Studies of PrP knockout mice have shown increased levels of  $\beta$ -amyloid(Parkin et al., 2007). It has been suggested on the basis of this that the expression of PrP inhibits the activity of BACE-1 but does not alter the expression levels of APP. In contrast to these findings, other work has suggested that PrP-knockout mice have decreased expression levels of APP (Kralovicova et al., 2009). The level of expression of PrP in cells has been shown to alter the expression of a number of metalloproteins and to alter copper metabolism. The expression and cell surface trafficking of PrP itself is also metal dependent(Haigh et al., 2005). APP, similar to PrP, binds copper and its trafficking at the cell surface are regulated by the levels of metals in cells(Acevedo et

al., 2011). While immunoprecipitation studies have suggested that PrP and BACE-1 directly interact (Parkin et al., 2007), any relation between APP expression and cleavage and PrP might be indirect due to the fact that they have similar regulatory pathways.

We investigated whether PrP and BACE-1 directly interact using isothermal titration calorimetry (ITC) and immunoprecipitation. Our findings did not demonstrate any evidence of a direct interaction. While BACE-1 showed strong binding of copper in ITC experiments, under similar conditions it did not bind PrP. Using promoter reporter constructs we demonstrated that PrP expression decreases the activity of the BACE-1 promoter. We also showed that altering PrP expression levels alters BACE-1 expression. Lastly, we have shown that increased PrP expression increases APP cleavage. This data suggests that there is no direct interaction of PrP and BACE-1. The implication is that the artificial depletion of PrP from cells alters cellular metabolism in a way that increases BACE-1 expression but this is possibly due to altered cellular metal metabolism rather than any direct relation between PrP and BACE-1.

## **METHODS**

Unless indicated, all reagents were purchased from Sigma.

### **Cloning of BACE-1, Protein Expression and Purification**

Mouse BACE-1 was cloned from cDNA reverse transcribed from mouse brain mRNA. The mature form of the protein (codons 46-501) was amplified from the cDNA using primers TagcatatgGAGACCGACGAGGAATCG (forward) and TagaagcttTTATTACTTGAGCAGGGAGATG (reverse) and cloned into the pET5a vector (Invitrogen) between the NdeI and HindIII sites of the multiple cloning site. An additional stop codon was also inserted to ensure thorough mRNA termination. The construct and the inset were verified by DNA sequencing. The immature form of BACE-1, Pro-BACE-1 (codons 22-501) was cloned in a similar way.

The plasmid was transformed into BL21 Codon-Plus (DE3)-RIL *E. coli* (Agilent). Large cultures of the bacteria were generated, and during the linear phase of growth protein expression was induced with 1 mM IPTG. Inclusion bodies were isolated from the bacteria and the protein denatured with 8M urea in 100 mM NaCl and 100 mM Tris pH 8. The protein was purified using copper based immobilised metal affinity chromatography (IMAC) using chelating sepharose (GE Healthcare). The purified protein was verified as BACE-1 using a specific antibody (Rabbit, C-terminal 486-501, Calbiochem) and western blot. The purified protein was refolded from urea and dialysed extensively to remove contaminants.

Recombinant mouse PrP was generated in similar way as previously described (Davies et al., 2009). Proteins were concentrated to the desired concentrations by gas pressure concentration and using PM30 membranes (Millipore).

### **Isothermal Titration Calorimetry**

Isothermal titration calorimetry (ITC) experiments were carried out on a Microcal VP-ITC. A series of injections of ligand were made into an isolated chamber containing BACE-1 at a constant temperature of 25°C. Heat changes within the cell were monitored during each injection of metal and recorded as the total heat change per second over time. A binding isotherm was then fitted to data and expressed as heat change per mole of ligand against the ligand to protein ratio. From this data, a model was used to predict the number of ligand binding sites on the protein involved in the reaction, the association constants of the binding ( $K_a$ ). Reactions were carried out at a range of pHs buffered with 50 mM MOPS. Different concentrations of ligand were used to ensure that results were repeatable over a variety of conditions. For protein protein interactions PrP was concentrated and introduced to BACE-1 with the titration needle. Analysis of the data was with Microcal's Origin based software.

Copper solutions were prepared by adding copper sulphate to 50mM buffer (as above) along with glycine in a molar ratio of 1:4. Glycine chelates copper, forming a  $\text{Cu}(\text{gly})_2$  complex. The excess glycine thus ensures that there is never any free copper in the reaction cell, avoiding the complications such as precipitation, formation copper hydroxide or free radicals. Buffer was added to the metal to ensure that the strong acidity of copper sulphate did not overwhelm the buffering capacity of the protein solution. The use of buffer in only one of the solutions would result in



significant background noise so therefore both protein and metal solutions were buffered. The concentration of copper used was dependent on the concentration of protein in the reaction cell. The method used for ITC analysis was as previously described (Davies et al., 2011). Data was analysed using previously described models (Hong et al., 2008; Hong and Simon, 2009) with Igor Pro software.

## **Mice**

Prion protein transgenic mice used in these studies were a gift from Professor Charles Weissmann (Zurich). The mice were either PrP-knockout mice (Bueler et al., 1992) or Tga20 mice overexpressing the prion protein 10-20 fold (Fischer et al., 1996). Both strains of transgenic mice were re-derived on a pure 129Sv background (Charles Weissmann, personal communication). These mice were used by us in a previous publication (Kralovicova et al., 2009). The wild-type control mice used for experiments using these lines was 129Sv (Harlan).

## **Western Blot**

Brain extracts were prepared by homogenising brains in a buffer containing phosphate buffered saline (PBS) pH7.5, 0.5% triton X-100 and 0.1% Igepal. Extracts were cleared of debris by centrifugation and the protein concentration quantitated using a BCA (bicinchoninic acid) assay according to the manufacturer's instructions (Sigma). Proteins were separated using polyacrylamide gel electrophoresis. The gel was run at 35 mA, 100W for 50 minutes. Proteins were then transferred onto a nitrocellulose membrane (Millipore) using a semidry transfer unit (Biorad). Membranes were blocked in 5% milk powder in PBS-T (0.1% Tween 20 in PBS). The membrane was incubated with primary antibody of the appropriate concentration for 1 hour. The

membrane was then washed 3-times for 10 minutes on a rocker with PBS, incubated with an appropriate horse radish peroxidase conjugated secondary antibody (Dako) for 1 hour and washed 2x 2 minutes, 15 minutes and 3x5 minutes with PBS-T. Membrane was then incubated with ECL Plus reagent (Amersham Biosciences) for 5 min. The signal was detected on X-ray paper (Amersham Biosciences). The membrane was stripped by washing in methanol for 10 seconds, washing in stripping buffer (25 mM Glycine, 1% w/v SDS, pH 2.0) for 1 hour and washing in TBS-T for 5 minutes. The membranes were then used for reprobing with other antibody. The intensity of signal was measured by using ImageJ software.

### **Immunoprecipitation**

A single mouse brain was homogenised in PBS pH7 with 0.1% Igepal. For each immunoprecipitation 1 µg of a monoclonal antibody was added and rocked on ice for one hour. 10 µg of pre-swelled protein-A sepharose was then added and incubated for a further hour. The sepharose was pelleted with centrifugation and washed with PBS four times. The bound protein was eluted with glycine pH 4.0 and immediately neutralised with 100 mM tris pH 7.0. BACE-1 was immunoprecipitated with Chemicon monoclonal 61-3E7 while PrP was immunoprecipitated with the mouse monoclonal antibody ICSM18 (DeGen). Following western blot, immunodetection was with a rabbit polyclonal for either BACE-1 C-terminal (486-501, Chemicon) and N-terminal (EE-17, Sigma), or for PrP as previously described (Kralovicova et al., 2009). The appropriate HRP conjugated secondary antibody (Dako) was then used for detection.

### **Cell Culture**

The cells used for these studies were either mouse N2A neuroblastoma cells or human SH-SY5Y cells. Some studies were also carried out using the fusion cell lines, F14 and F21 (Holme et al., 2003). All tissue culture reagents and plastic-ware were obtained respectively from Lonza Biologics plc (Slough, UK) and Greiner Bio-One Ltd (Stroudwater, UK). SH-SY5Y cells were maintained in a 50/50 mix of DMEM and HAMS F12 with 10% fetal calf serum in 75 cm<sup>2</sup> flasks at 37°C (5% CO<sub>2</sub>). N2A, F14 and F21 cell lines were maintained under similar conditions except that the medium used was DMEM 10% fetal calf serum. Stable cell lines overexpressing PrP were achieved by transfection of the cell lines with pCDNA3.1 containing the open reading frame of mouse PrP. This construct and mutants of this were as previously described (Haigh et al., 2005). Transfected cells were maintained in G418 (PAA) 800 µg/ml to select for cells carrying the plasmid.

### **Promoter Studies**

The promoter studies were carried out using a luciferase reporter system. The rat BACE-1 promoter reporter was as previously described (Lange-Dohna et al., 2003). 1.5 kB upstream of the ATG start codon was cloned into the pGL3-Basic vector (Promega). The human BACE-1 promoter was cloned into pGL3-Basic by amplification from a Bacmid clone (RP11-677N11, ImaGenes) of a 4.3 kb fragment beginning at -4372 of the promoter sequence.

Transfections into N2A, SH-SY5Y or F14 cells were performed in 24-well plates seeded at 5x10<sup>4</sup> cells/well 24 h prior to transfection. Transfections were performed using FuGENE6® transfection reagent (Roche) as per manufacturer's instructions. To control for variation in transfection efficiency among replicates, promoter constructs

were co-transfected with the *Renilla* luciferase vector, pRL-TK or pRL-SV40, (Promega). At 48 h post-transfection, cells were harvested and firefly and *Renilla* luciferase chemiluminescence were measured using the Dual-Luciferase® Reporter Assay System (Promega) in a BMG Fluostar Omega platereader (BMG Labtech GmbH). Luciferase activity was calculated as the ratio of firefly to *Renilla* luciferase activity. Each construct was tested in triplicate with at least three independent transfection experiments.

### **APP Cleavage Assay**

The luciferase based reporter assay was as previously described (Hoey et al., 2009). The assay is based upon activation of a firefly luciferase reporter construct pFR under the control of a synthetic promoter consisting of five tandem repeats of the yeast GAL4 activation sequence upstream of a minimal TATA box. Activation of the reporter results from binding of the GAL4- DNA binding domain. This domain is present, fused to the C-terminus of APP695 when expressed in cells from a pRC-CMV vector. The vector contains a cDNA encoding for human APP695 fused in-frame at its C terminus to the GAL4 DNA-binding domain. Cleavage of overexpressed APP by secretases results in the release of ACID domain of APP fused to GAL-4 which can then bind to the pFR vector. Luciferase activity relative to cell density was determined by co-transfection with a renilla luciferase vector driven by the HSV thymidine kinase (TK) promoter (pRL-TK). Luciferase activity was measured using the Promega Dual-Luciferase Report Kit as per the manufacturer's instructions. Assays were carried out using a Fluostar Omega plate reader (BMG). In some experiments the activity of beta-secretase was inhibited by the addition of 100 nM of amyloid precursor protein beta-secretase inhibitor (Calbiochem).



## **RESULTS**

### **Immunoprecipitation of BACE-1**

A previous study has suggested that PrP and BACE-1 interact directly based on immunoprecipitation of PrP (Parkin et al., 2007) from SH-SY5Y cells. We undertook a similar investigation but with protein extracts from the brains of transgenic mice. The advantage of these transgenic mice is that immunoprecipitation can also be performed on mice overexpressing PrP or knocked out for PrP. As overexpressing mice express much higher levels of PrP immunoprecipitation of BACE-1 should increase the likelihood of co-precipitating PrP along with it if the two proteins interact. Additionally, PrP-knockout mice lacking PrP control for non specific co-precipitation artefacts. In Figure 1 immunoprecipitation of BACE-1 with a specific monoclonal antibody to the C-terminus resulted in immunoprecipitation of BACE-1 from wild-type as well as PrP-knockout and PrP-overexpressing mice as determined by western blot. However, analyses of the immunoprecipitated material with an antibody to PrP did not show any detectable trace of PrP. This suggests that PrP does not co-precipitate with BACE-1.

Additional immunoprecipitation experiments were carried out with a PrP monoclonal antibody. This resulted in specific immunoprecipitation of PrP from wild-type and PrP-overexpressing mouse brain extracts but not from PrP-knockout mice. The antibody detected a band of 72 kD in wild-type brain corresponding to the mature glycosylated form of BACE-1. Analysis of the immunoprecipitants for the presence of BACE-1 showed trace amounts of a band the same size as BACE-1 in extracts from both wild-type and PrP-knockout mouse brains (Figure 1). However, the presence of equivalent levels of the band in extracts varying in PrP expression levels suggest this

band is not due to co-precipitation and is a non-specific artefact. As it was previously suggested that PrP and BACE-1 interact via the pro-domain (Griffiths et al., 2011), we tested further for the presence of pro-BACE-1 in the immunoprecipitations using the EE-17 antibody originally used to suggest the interaction. This antibody detected an additional band at 60 kD in wild-type brain which was smaller than the mature band at 72 kD because of the incomplete glycosylation. However, we were unable to detect any co-precipitated BACE-1 of any size with this antibody. These data do not support the notion that PrP and BACE-1 directly interact.

### **ITC Analysis of Ligand Binding to BACE-1**

We have previously used ITC to analyse the binding of copper to a range of proteins including PrP and the synucleins. BACE-1 is also reported to bind copper via its intracellular domain. We used ITC to analyse copper binding to BACE-1. As can be seen in figure 2. Copper sulphate as a glycine chelate was titrated into 100  $\mu$ M BACE-1 at pH7 (50 mM MOPS). Binding of copper to BACE-1 produced an endothermic isotherm indicating a strong and specific binding of copper to BACE-1. Analyses indicated a single binding event with stability constant of  $2.3 \times 10^{11} \text{ M}^{-1}$ .

Having verified that we can observe binding of a known ligand to BACE-1 we also attempted to observe binding of PrP to BACE-1 using ITC. Concentrated PrP was titrated into a solution of BACE-1. Various experiments were attempted with different pH (pH5-pH9). We did not observe a positive ITC isotherm under any of the conditions tested (Figure 2). We also did not observe binding when ITC was performed using the immature Pro-BACE-1. As ITC can detect protein-protein

interactions with affinity values as low as  $10^2$  M<sup>-1</sup> our findings suggest that the two proteins do not bind to each other.

### **Altered PrP Expression Influences BACE-1 Protein Levels**

We have previously observed that altered PrP levels can have a dramatic effect on the expression of other proteins, especially other copper binding proteins and antioxidants (Kralovicova et al., 2009). Protein extracts were prepared from the brains of age matched transgenic mice. Using the western blot technique and immunodetection with a specific antibody we looked at the expression of BACE-1 in the brains of mice with different expression levels of PrP. The findings indicated an inconsistent relation between protein expression levels of BACE-1 and PrP. Both knockout and overexpression of PrP both increased the levels of BACE-1 (Figure 3).

### **Increased PrP Expression Alters BACE-1 Promoter Activity**

Analysis of protein expression of BACE-1 under different levels of PrP expression suggests an indirect relationship between the levels of PrP expression and BACE-1 expression. In order to try and obtain a clearer picture of how PrP expression could alter BACE-1 expression we looked at the effect at the level of gene transcription using a variety of different cell lines with different levels of PrP expression. We transfected these cell lines with a luciferase reporter construct driven by a 1.5 kb fragment of the rat BACE-1 promoter (Lange-Dohna et al., 2003). Comparing N2A cell lines either overexpressing PrP or transfected with the empty vector we observed a clear and significant decrease in the level of BACE-1 promoter activity (Figure 4A). We previously created a PrP knockout cell line and parallel derived PrP expressing cell line by fusing cerebellar granule neurons to a neuroblastoma cell line ((Holme et



al., 2003)). These fusion cell lines have a similar phenotype. Transfecting these cell lines with the BACE-1 promoter reporter resulted in a similar finding. BACE-1 promoter activity was higher in the PrP knockout cell line when compared to the wild-type (Figure 4B). As species specific difference might have had an impact on our results we also looked at the human SH-SY5Y cell line. In this case we also used a reporter construct with a 4 kb fragment of the human BACE-1 promoter. In this case SH-SY5Y cells overexpressing PrP showed decrease activity of both the rat and human BACE-1 promoter (Figure 4C). Therefore, regardless of cell type, promoter origin of background PrP levels, increased PrP expression reduced BACE-1 promoter activity.

### **Increased PrP Expression Increases APP Cleavage**

Study of both BACE-1 transcription and protein expression levels did not provide a clear picture of the relation between the expression of PrP and BACE-1. We therefore used an APP cleavage luciferase reporter assay to look at the rate of cleavage of APP under different PrP expression conditions. In this assay APP695 was expressed as a C-terminal fusion product with the GAL-4 DNA binding domain. Cleavage of APP, releasing the ACID domain resulted in activation of a luciferase reporter by the GAL-4 fragment binding to the yeast GAL-4 promoter activation sequence driving expression of the firefly luciferase. This report assay has been shown previously to preferentially report changes in BACE-1 driven APP cleavage (Hoey et al., 2009). We looked at the activity of this assay in two different cell lines overexpressing PrP, the N2A cell line and the F14 PrP-knockout fusion cell line. In both cell lines the overexpression of PrP resulted in increased cleavage of APP (Figure 5). In order to verify that the measured increase activity in the assay was as a result of increased

beta-secretase activity we tested the response in the assay to the presence of a known beta-secretase inhibitor. In N2A cells 100 nM of the peptide inhibitor inhibited the assay  $84 \pm 3$  % in control cells and  $87 \pm 5$  % in PrP overexpressing cells ( $n=4$ , Student's t test,  $p < 0.05$ ) This indicates that the inhibitor blocked the majority of activity measured in the assay regardless of the condition and clearly demonstrates that activity in the assay is a direct measure of the activity of beta-secretase. This implies that overexpression of PrP results in increased production of the AICD fragment as a result of increased BACE-1 activity. Additionally, we also overexpressed in both cell lines, two mutants of PrP. The first was a deletion of the octameric repeat region known to bind copper (PrP $\Delta$ 51-89) and the second was a mutant with the palindromic AGAAAAGA motif (PrP $\Delta$ 112-119). Both mutants showed decreased internalisation while only the PrP $\Delta$ 51-89 is unable to bind copper (Haigh et al., 2005). Deletion of the copper binding domain abolished the increase in APP cleavage from PrP overexpression while PrP $\Delta$ 112-119 did not (Figure 5). This implies that the effect of PrP on APP cleavage may be dependent on its ability to bind copper.

## **DISCUSSION**

A potential role of PrP in regulating susceptibility to other diseases in addition to its role in prion diseases has sparked considerable interest in recent years. However, investigating such a role should not be carried out without consideration of what is known the cellular role of PrP. PrP is a copper binding protein and has been suggested to function as an antioxidant (Brown et al., 1997; Brown et al., 1999). The redox chemistry as well as the affinity and co-ordination of copper binding have all been investigated (Burns et al., 2003; Davies et al., 2009; Klewpatinond et al., 2008; Walter et al., 2006). Copper binding has also been shown to alter the cellular trafficking of PrP(Haigh et al., 2005) and alterations in expression of PrP have been shown to alter copper metabolism and to alter the expression of other known metalloproteins and antioxidants(Kralovicova et al., 2009). In this regard, it is not surprising that altering PrP expression alters BACE-1 expression as this protein also binds copper necessary for its activity as a secretase(Angeletti et al., 2005).

A role for PrP in Alzheimer's disease has been suggested from two lines of evidence. The first is the suggestion that PrP can act as a receptor for beta-amyloid(Lauren et al., 2009). As a cell surface glycoprotein with a strong hydrophobic domain the potential for it to interact with other proteins is very high but this could also result in artefactual interactions. PrP has been suggested to have a large number of binding partners and most of these are probably non-specific interactions. The suggestion that PrP binds beta-amyloid has also been challenged (Calella et al., 2010), and may also just be a result of the test conditions rather than a reflection of what occurs during Alzheimer's disease. The second link between PrP and Alzheimer's disease has been more indirect. In this case, it was suggested that PrP could interact with BACE-1 and

alter the extent of APP cleavage and beta-amyloid generation (Parkin et al., 2007). This initial report suggested that PrP-knockout mice have increased levels of beta-amyloid. The hypothesis presented was that PrP expression decreased BACE-1 protein interaction with APP with no change in the cellular levels of the BACE-1 protein. A further study suggests that the pro- domain of the immature BACE-1 interacts with PrP in the Golgi. This interaction prevents BACE-1 reaching the surface of the cell and thus limits APP cleavage (Griffiths et al., 2011). Furthermore, it was suggested that when the APP expressed contains the Swedish mutation, that APP is cleaved preferentially in Golgi where BACE-1 has accumulated.

We began this investigation wishing to build on such findings. Unfortunately, we were not able to verify any of the previous data. Indeed, our findings support a notion the opposite of that previously suggested. In particular, we have shown with several systems that PrP alters the expression levels of BACE-1 and that the level of APP cleavage is increased in cells overexpressing PrP. The implications of our findings are that PrP does not decrease APP processing and that any such changes have little to do with the direct interaction of PrP and BACE-1, but are more likely to be related to altered expression and metabolism of the protein.

The cleavage of BACE-1 from its prodomain is not necessary to activate BACE-1 (Capell et al., 2000; Cole and Vassar, 2008). Although the activity of the unprocessed precursor is less, this would not necessarily result in a lower rate of APP cleavage. Therefore, even if PrP did interact with this domain it would not necessarily result in reduced activity. The cellular trafficking of BACE-1 is quite complex and involves retrograde transport as well as possible transport between the Golgi and

endosomes (Finan et al., 2011). Additionally, while it is known that APP cleavage can occur at the cell surface, there is also increasing evidence that this can occur elsewhere, particularly the endosomes (Sannerud et al., 2011). Therefore, a reduced level of BACE-1 at the cell surface does not necessarily imply that this will result in decreased cleavage of APP.

Our analysis of a potential interaction of BACE-1 and PrP provided no evidence to support the theory. Both immunoprecipitation studies and ITC studies failed to show any evidence of a direct interaction. While it has been suggested the interaction requires the pro-domain our ITC data shows no difference between Pro-BACE-1 and the mature form. However, if the interaction was mediated by this domain, some evidence should have been apparent from the immunoprecipitations. The published evidence for this interaction comes from a mixture of immunoprecipitation experiments and Surface Plasmon Resonance (Griffiths et al., 2011). The proposed dissociation constant for the interaction is in the low micromolar range which is quite low and given the relatively low expression of BACE-1 might not be sufficient for the interaction to be noticeable *in vivo*. Furthermore, it is unclear what percentage of BACE-1 present *in vivo* exists with the prodomain still intact. Cleavage of this domain occurs very early during its synthesis (Capell et al., 2000), and even if PrP binds to this there is no indication that this prevents cleavage. As mentioned above, BACE-1 is recycled and it is likely that the vast majority of the protein present in the cell is in the mature form. Therefore any interaction of PrP with the prodomain is likely to have little consequence for the overall activity of the protein.

Previous work on this issue also suggested that altered PrP levels do not alter the level of expression of APP (Parkin et al., 2007). We have previously analysed APP levels in PrP transgenic mice. Western blot analysis of APP expression in the brains of PrP-knockout mice showed much lower levels than in wild-type (Kralovicova et al., 2009). APP expression was slightly increased in mice overexpressing PrP. We initially interpreted this finding as indicating reduced expression of APP in the absence of PrP. An alternative possibility is that APP turnover in PrP-knockout mice is rapidly increased. Increased turnover is likely to result in increased degradation of APP through different pathways including the amyloidogenic pathways. This would explain the previous noted increase in  $\beta$ -amyloid levels in PrP knockout mice. We also measured increase expression of BACE-1 in the same mice but the complete loss of APP would also require increased metabolism of the larger soluble APP fragment as well.

Although most of our data suggests that increased PrP expression results in increased BACE-1 expression and increased activity of the protein, the results from the promoter studies appear to contradict this. While the measured decrease in promoter activity was robust through different conditions, it is unclear what the impact of this would be on protein levels in the cell. As on one hand PrP overexpression appears to increase BACE-1 protein levels in the cell but on the other hand decrease translation from the BACE-1 gene, it is possible that overexpression of PrP has its effect by decreasing BACE-1 turnover. This in turn could have a feed-back effect on the promoter inhibiting its activity because of the higher levels of protein retained by the cell. Additionally, the data from the transgenic mice suggests that both knockout and overexpression of PrP increase BACE-1 levels. In the case of overexpression,

stabilisation of protein levels as suggested might be the cause. However, in the case of complete PrP loss, an alternative mechanism might increase BACE-1 levels. We have observed increased promoter activity or it could result from other downstream events as a consequence of lost PrP expression. Altered copper metabolism and increase levels of oxidative stress are both known to be a consequence of loss of PrP expression (Brown, 2005; Kralovicova et al., 2009). Alternatively, the increased levels of BACE-1 in PrP overexpressing mice might be a consequence of increased expression of PrP in regions where it would not normally be expressed. Thus in regions where PrP is not normally expressed this would result in a different response by BACE-1 regulatory systems.

The assay measuring levels of APP cleavage was a direct measure of AICD formation (Hoey et al., 2009). Once released, this domain is then free to bind to the reporter plasmid and induce luciferase expression. The increased expression of PrP increased this reporter activity, implying increased cleavage of APP. A mutation in PrP that we have showed results in loss of copper binding blocked this effect. While it could be argued that this is because of altered PrP trafficking, another mutation known to alter PrP internalisation did not have the same effect. This implies that the ability of PrP to increase APP cleavage is dependent on its copper binding ability. Therefore, it is likely that the impact of altered PrP expression on APP cleavage and possibly BACE-1 activity and expression is as a result of altered copper metabolism. Altered PrP levels alter copper levels in cells and the expression of a range of copper binding proteins as already mentioned. Therefore, while it might be interesting to postulate a potential role of PrP in a disease other than prion disease, it is important to consider that there are many indirect effects that change protein expression and

artificial manipulation of one protein can have apparent consequences which could easily be misinterpreted if the bigger picture (eg. a common association with copper metabolism) is not considered.

The formation of beta-amyloid is also influenced by other copper binding proteins such as CCS (Gray et al., 2010). Again, this effect is suggested to be mediated through BACE-1 and possibly as a result of either altered copper availability for the protein or protein trafficking.

In summary, we have investigated the interaction of BACE-1 and PrP at a variety of levels from the genetic to the level of activity of BACE-1 in APP cleavage. Our data do not suggest that PrP plays a protective role, decreasing beta-amyloid formation and do not support the notion that the two proteins interact. It is more likely that the two proteins and possibly APP are regulated by common mechanisms because of their associations with copper metabolism as all three proteins bind copper. Therefore, we feel that our data does not provide any evidence for an association between PrP and Alzheimer's diseases. A recent study of genetic correlations looking at the Prnp gene and both Alzheimer's disease and Creutzfeldt-Jacob disease found no association between Prnp variance and Alzheimer's disease (Calero et al., 2011).

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## FIGURE LEGENDS

### Figure 1 Immunoprecipitation Experiments

Specific monoclonal antibodies were used to immunoprecipitate either BACE-1 or PrP from protein extracts derived from the brains of mice. The mice were either wild-type mice or age matched PrP-knockout mice (KO) or PrP-overexpressing mice (OE). The immunoprecipitated material was electrophoresed on a PAGE gel and transferred to a membrane by western blot. As a positive control brain extract from wild-type mice (Con) was also included on the blot. To confirm that the immunoprecipitation resulted in isolation of BACE-1 or PrP from the extracts immunodetection was carried out with polyclonal antibodies (top blot for each). In the case of BACE-1 (top) bands in all lanes can be observed indicating equivalent amounts of protein were immunoprecipitated. In the case of PrP no protein is present in the KO lane as would be expected, but protein is present in both WT and OE lanes. The OE lane shows much higher levels due to the 10-20 fold higher levels of PrP as reported in the literature. Possible co-precipitation of PrP with BACE-1 was assessed with the PrP specific antibody but no band was observed in any lane except the brain extract control. Co-precipitation of BACE-1 with PrP was also assessed (bottom) using two different antibodies where the epitope was either in the C- or N-terminus. For the BACE-1 C terminus antibody although a faint band is present in the KO and WT lanes this is not likely to relate to co-precipitation with PrP as PrP was absent from the KO lane suggesting that BACE-1 does not immunoprecipitate with PrP. Analysis of the PrP immunoprecipitations with the N-terminal BACE-1 antibody EE-17 used previously (Griffiths et al., 2011) showed no detectable bands for WT, KO or OE confirming this finding.

### **Figure 2 Ligand Binding to BACE-1**

ITC was used to assess any potential interaction between BACE-1 and PrP. Analysis were carried out with BACE-1 at 100  $\mu$ M pH7. **A** As a positive control copper binding to BACE-1 was measured on titration of copper sulphate as a glycine chelate. A positive isotherm was measured indicating strong binding. **B-C** PrP was titrated into the BACE-1 solution to result in a 2.5:1 molar ratio (ie PrP final concentration was 250  $\mu$ M). However, no isotherm was obtained indicating that no binding was observed for either the mature BACE-1, 46-501 (B) or pro-BACE-1, 22-501.

### **Figure 3 Western Blot**

**A** The level of expression of BACE-1 protein was assessed using western blot and immunodetection with a specific antibody. Protein extracts from the brains of age matched wild-type, PrP-knockout (KO) and PrP-overexpressing (OE) mice were prepared and 50 ug of total protein was loaded onto a PAGE gel and electrophoresed. After western transfer the relative levels of BACE-1 were detected by immunodetection and chemiluminescence. The blots were stripped and reprobed with a tubulin specific antibody to assure equivalent protein loading. **B** Densitometric analysis was carried out on blots from four separate experiments and the mean band density relative to the wild-type value plotted with standard error. Both KO and OE values were both significantly higher than WT (Student's t test,  $p < 0.05$ ).

### **Figure 4 Promoter Activity**

A luciferase reporter assay was used to assess the relative activity of the rat BACE-1 promoter in different cell lines expressing different levels of PrP.

**A** The N2A cell line was stably transfected to over-express mouse PrP and control cells were transfected with the empty vector (pCDNA). When transfected with the report for BACE-1 promoter activity the activity of the promoter in the PrP transfected cells was significantly lower than for the control cells.

**B** The stable cell fusion line F14 was generated from PrP knockout mouse cerebellar granule cells. A similarly derived line (F21) expresses wild-type levels of PrP. When comparing the activity of the BACE-1 promoter luciferase reporter in these cell lines, activity was significantly higher in F14 cells when compared to F21.

**C** The human SH-SY5Y cell line was stably transfected to overexpress PrP or with the empty vector (pCDNA). Luciferase reporter constructs for both human and rat BACE-1 were used to compare the effects of increased PrP levels. In both cases, increased PrP expression resulted in a significant decrease in the activity of the reporters when compared to control. Shown are the mean and standard error for at least three separate experiments. Significant difference was determined with the Student's t test ( $p < 0.05$ ).

### **Figure 5 APP Cleavage Assay**

A lucifase based assay that reports cleavage of APP was used to determine the effect of altered PrP expression on APP cleavage. The assay is based upon binding of a GAL-4 DNA binding domain to the DNA sequence in the luciferase reporter construct. Binding is directly proportional to release of the ACID domain from APP to which the GAL-4 domain is fused. Both **A** N2A and **B** F14 cells overexpressing mouse PrP were used. When compared to controls transfected with pCDNA, cells transfected with PrP showed significantly increased levels of luciferase activity suggesting increased levels of APP cleavage. Additional experiments were performed

with N2A and F14 cells transfected with two PrP mutants. The first mutant (MM) lacks the octameric repeat region and does not bind copper while the second mutant (NoInt) lacks the palendromic region (amino acid residues 112-119). Both mutants alter internalisation of PrP but only MM does not bind copper. While the NoInt mutant was not significantly different from wild-type PrP in its effect on APP cleavage, the MM mutant was not significantly different from pCDNA suggesting that lack of copper binding to PrP blocks the effect of PrP on APP cleavage. Shown are the mean and standard error of n=5 for N2A cells and n=4 for F14 cells. Significant difference was determined with the Student's t test ( $p < 0.05$ ).

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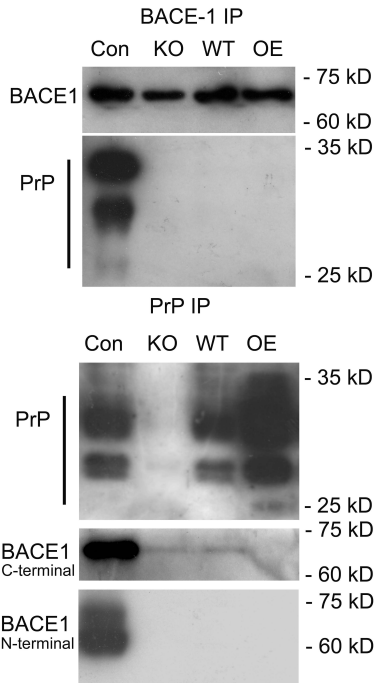


Figure 1



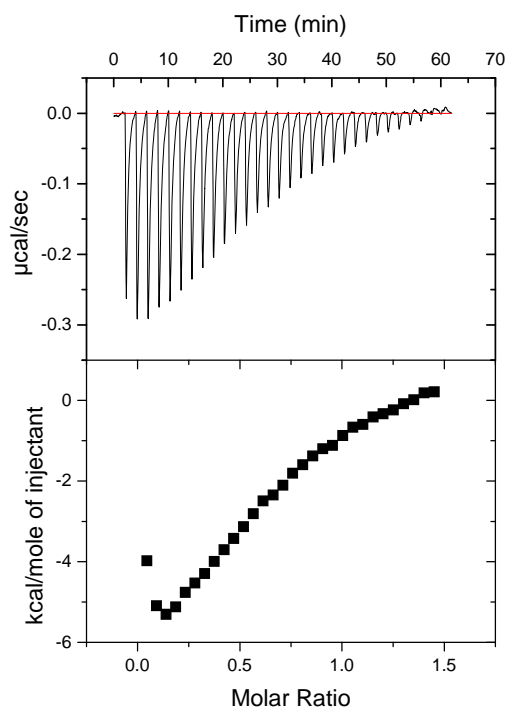
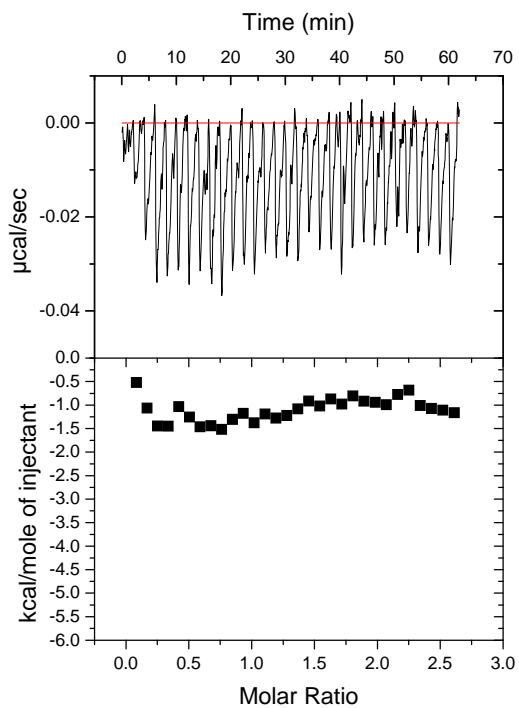
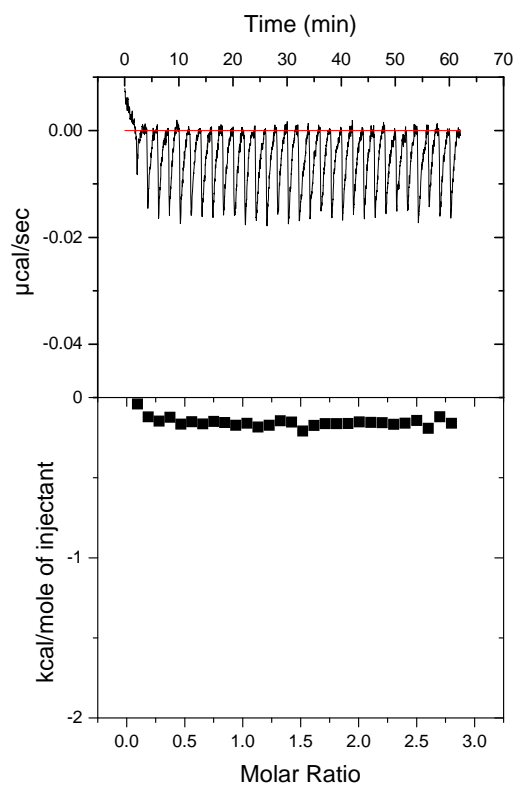
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Figure 2

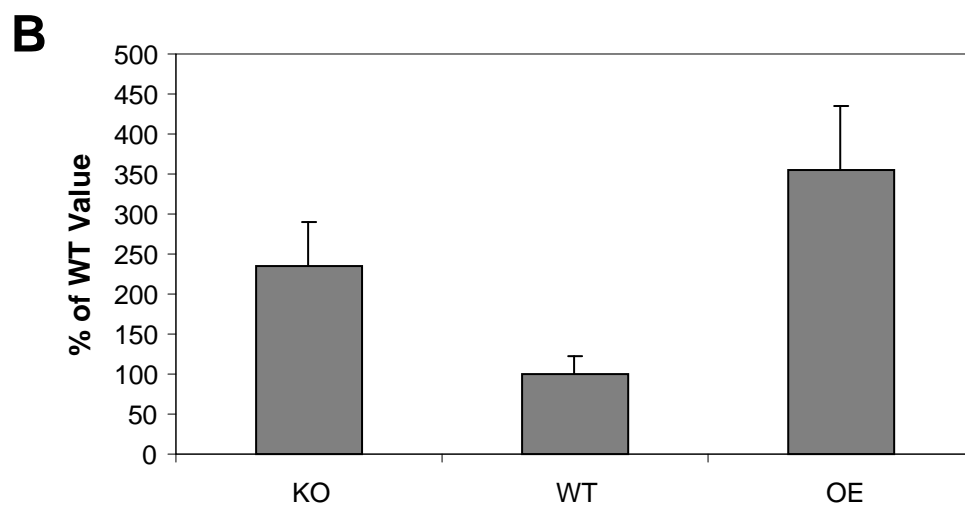
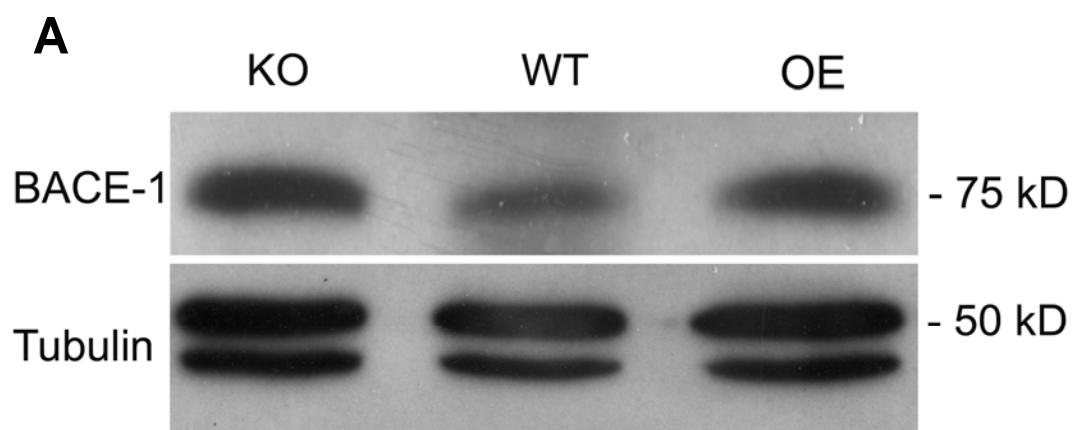


Figure 3

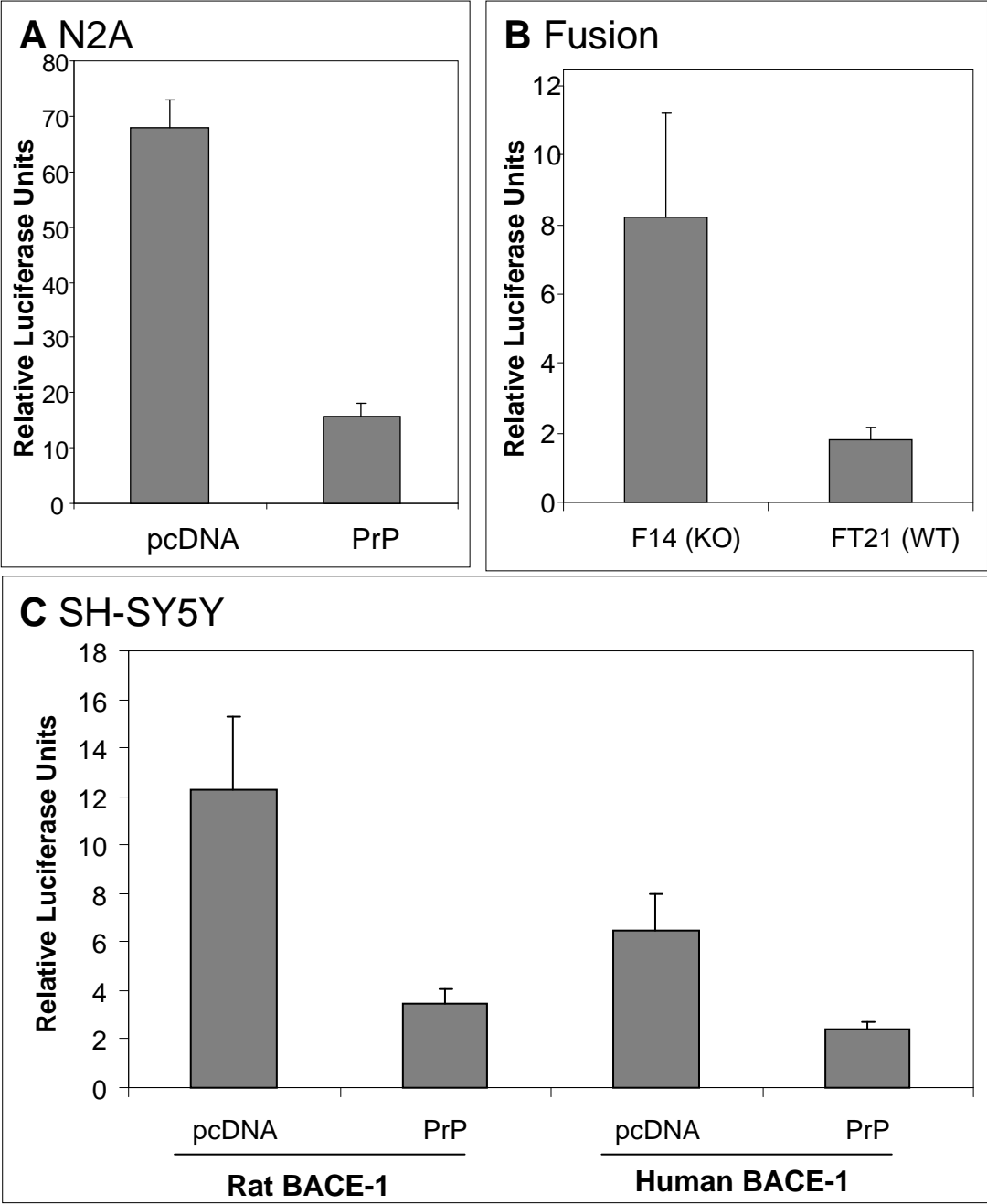


Figure 4

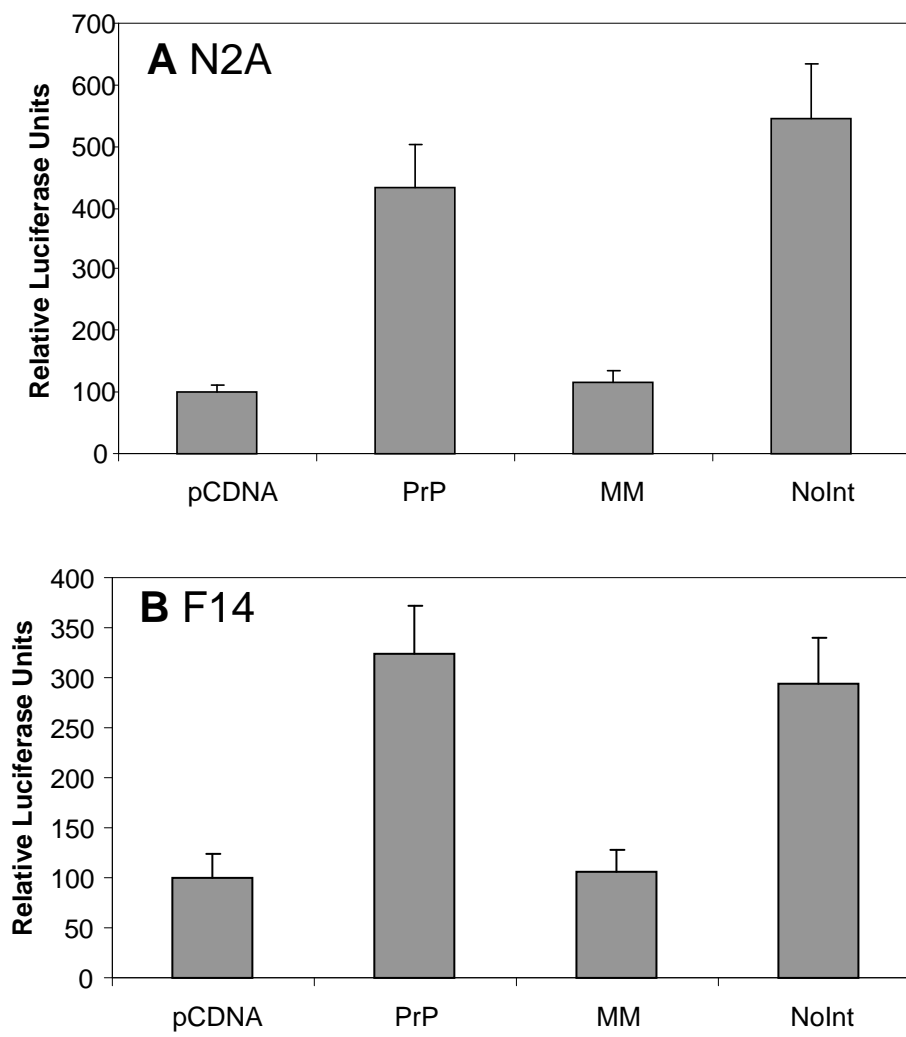


Figure 5